Rabies Virus Pseudotyped with CVS-N2C Glycoprotein as a Powerful Tool for Retrograde Neuronal Network Tracing

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Abstract

Background: Efficient viral vectors for mapping and manipulating long projection neuronal circuits are crucial in brain structural and functional studies. The glycoprotein gene-deleted SAD strain rabies virus pseudotyped with the N2C glycoprotein (SAD-RV(ΔG)-N2C(G)) shows high neuro-tropism in cell culture, but its *in vivo* retrograde infection efficiency and neuro-tropism have not been systematically characterized.

Methods: SAD-RV(ΔG)-N2C(G) and two other broadly used retrograde tracers, SAD-RV(ΔG)-B19(G) and rAAV2-retro were respectively injected into the VTA or DG in C57BL/6 mice. The neuron numbers labeled across the whole brain regions were counted and analyzed by measuring the retrograde infection efficiencies and tropisms of these viral tools. The labeled neural types were analyzed using fluorescence immunohistochemistry or GAD67-GFP mice.

Result: We found that SAD-RV (ΔG)-N2C (G) enhanced the infection efficiency of long-projecting neurons by ~ 10 times but with very similar neuro-tropism, compared with SAD-RV (ΔG)-B19(G). On the other hand, SAD-RV(ΔG)-N2C(G) showed comparable infection efficiency with rAAV2-retro, but had a more restricted diffusion range, and broader tropism to different types and regions of long-projecting neuronal populations.

Conclusions: These results demonstrate that SAD-RV(ΔG)-N2C(G) can serve as an effective retrograde vector for studying neuronal circuits.

Key words： Viral vector, N2C Glycoprotein, Neuronal circuits, Retrograde tracing

Background

In the central nervous system, distinct brain regions work corporately through particular circuit connections to process different and complex information [1-6]. Neuronal

circuits are the keystone to brain functions and their anatomical and functional aberrations are closely related to many neurodegenerative diseases [7-9], such as Parkinson's disease [10, 11], Alzheimer's disease [12] and Huntington's disease [13]. Thus, it is critical to develop efficient tools for anatomical mapping and functional decoding of neuronal circuit connections.

Retrograde tracers, owing to their unique properties of entry at axon terminals and then being transported to the cell bodies, are useful tools for targeting the long-projecting neuronal circuit assemblies [14, 15]. Compared with the classical chemical tracers [16-20], viral vectors are able to deliver genetic elements to neuronal populations with specific projection properties or molecular features, and hence are superior in morphological visualization, activity monitoring and functional modulation in neuroscience studies. Nowadays, viral tools are drawing close attention from the field of neuroscience. The rabies virus (RABV) [21-24], herpes simplex virus (HSV) [25-29], canine adeno virus-2 (CAV-2) [30-32] and retrograde adeno associated virus (rAAV2-retro) [33] are among the most commonly recently used retrograde viral vectors. They seem to have rather different infection efficacies and tropisms, although not yet thoroughly explored. RABV and HSV have broader tropism for different types of neurons [27, 34], and much higher cytotoxicity compared with CAV-2 and rAAV2-retro. CAV-2 and rAAV2-retro are very valuable due to their low toxicity and outstanding retrograde infection efficiency, but are limited by the gene delivery capacity and heterogeneous tropism of different neurons [35, 36]. By far, RABV is reported to ensure robust gene expression, possess the most exclusive neuro-tropism and the broadest range of host species among the above mentioned viruses, but is limited by its cytotoxicity and retrograde infection efficiency. Recent studies have successfully attenuated [37] and even eliminated [35] its cytotoxicity. Moreover, RABV enveloped

with the N2C glycoprotein (N2C(G)) from the Challenge Virus Strain (CVS) displayed an increased neuro-tropism in cell culture [38] and trans-synaptic efficiency *in vivo* [39]. These improvements endow the RABV-N2C(G) with great potential in both structural and functional studies of neuronal circuits. However, since the cellular environment and receptors involved may be different, the higher *in vitro* neuro-tropism and *in vivo* trans-synaptic spread efficiency do not mean higher retrograde infection efficiencies. Thus, the *in vivo* retrograde infection efficiency and tropisms of the N2C(G) enveloped RABV to long-projecting neuronal circuits and the comparison with the two outstanding retrograde tracers (SAD strain RABV and rAAV2-retro) are still unknown.

To address these questions, in this study, we first enveloped the glycoprotein gene-deleted SAD-RABV with N2C(G) (SAD-RV(ΔG)-N2C(G)) or the native glycoprotein (SAD-RV(ΔG)-B19(G)), and then compared the *in vivo* retrograde infection properties of the SAD-RV(ΔG)-N2C(G) with that of SAD-RV(ΔG)-B19(G) and rAAV2-retro. We found that the SAD-RV(ΔG)-N2C(G) showed a much enhanced retrograde infection efficiency (over 10 times) than the SAD-RV(ΔG)-B19(G). Compared with rAAV2-retro, we established that, the SAD-RV(ΔG)-N2C(G) exhibited a broader tropism of different types of projection neurons in different upstream regions, and a more restricted diffusion range at the injection site with the comparable retrograde infection efficiency. These results demonstrate that the SAD-RV(ΔG)-N2C(G) can serve as a more effective retrograde tracer for studying input neuronal networks.

Methods

Animals

All surgical and experimental procedures were conducted in accordance with the guidelines of the Animal Care and Use Committees at the Wuhan Institute of Physics

and Mathematics, Chinese Academy of Sciences. Adult male C57BL/6 mice were purchased from Hunan SJA Laboratory Animal Company. GAD67-GFP transgenic mice [40] were gifts from Professor Shumin Duan (Zhejiang University), and bred with adult female C57BL/6 mice. All animals were fed *ad libitum* with food and water. A dedicated room with a 12/12 h light/dark cycle was used to house animals.

Virus Information

The viral vectors (SAD-RV(ΔG)-B19(G)-EGFP, SAD-RV(ΔG)-N2C(G)-EGFP, SAD-RV(ΔG)-N2C(G)-mCherry, rAAV2-retro-EF1α-EYFP, rAAV2-retro-EF1α-mCherry) were all packaged by BrainVTA Co., Ltd. (Wuhan, China) and all aliquots were stored at -80℃.

Production of BHK-N2C(G) cells

For the BHK-N2C(G) cell lines, FUGW-H2B-GFP-P2A-N2C(G) was created by inserting the N2C-glycoprotein gene (Addgene, # 73476) with histone GFP into the Lentivirus expression vector FUGW (Addgene, # 14883), then transfected into lentiviral packaging cells. After filtration, FUGW-H2B-GFP-P2A-N2C(G) was used to infect BHK cells.

Packaging of the SAD-RV(ΔG)-N2C(G)-EGFP

The SAD-RV(ΔG)-B19(G)-EGFP was packaged using standard methods as described in previous reports [41]. For the SAD-RV(ΔG)-N2C(G)-EGFP, BHK-N2C(G) cells were used to stably express N2C(G) for packaging SAD-RV(ΔG)-N2C(G)-EGFP. The SAD-RV(ΔG)-B19(G)-EGFP was used to infect BHK-N2C(G) cells for 48 hours. Then, after collecting viral supernatant, the BHK-N2C(G) cells were washed with PBS, digested with pancreatin, and amplified. After 48 hours, the viral supernatant of SAD-RV(ΔG)-N2C(G)-EGFP was collected, filtrated with filter (0.45 μm) and stored at -80℃. The concentration procedure of SAD-RV(ΔG)-N2C(G)-EGFP was the same as

previously reported [41].

The SAD-RV(ΔG)-N2C(G)-mCherry was obtained using the same procedure as the SAD-RV(ΔG)-N2C(G)-EGFP. All rabies viruses were titrated using the same procedure as previously reported [6] and stored at -80℃. The rAAV2-retro-EF1α-EYFP and rAAV2-retro-EF1α-mCherry were titrated using QPCR.

Stereotactic Surgery

Animals were anesthetized with chloral hydrate (400 mg/kg), and then placed in a stereotaxic apparatus (RWD, 68030, Shenzhen, China). The skull above the targeted areas was thinned with a dental drill (STRONG, Guangdong, China) and then removed carefully with a curved needle. A mixture of virus and CTB594 (Thermo Fisher Scientific, C34777; with the final concentration of 0.02 mg/ml) was injected into the target brain regions (VTA: a-p, -3.10 mm; m-l, ±0.50 mm, d-v, -4.50 mm, 200 nL volume; or DG: a-p, -1.70 mm; m-l, -0.90 mm; d-v, -1.95 mm, 100 nL volume) using an injector connected to a glass micropipette (WPI, 4878, United States), and driven by a syringe pump (Stoelting, Quintessential stereotaxic injector, 53311, United States). After injection, the glass micropipette was left in place for an extra 10 mins to minimize diffusion and then slowly withdrawn.

At last, animals were put back into the housing room after suture and the application of lidocaine hydrochloride on to the wound.

Slice Preparation and Confocal Imaging

Mice were anesthetized with an overdose of chloral hydrate (600 mg/kg), and then perfused transcardially with PBS followed by 4% paraformaldehyde solution (PFA, 158127MSDS, Sigma). The brain tissues were removed and post-fixed overnight in 4% paraformaldehyde solution at 4℃, then sectioned into 40 μm coronal slices with a cryostat microtome (Thermo Fisher, NX50, Germany).

The brain slices were selected (every sixth section of the whole brain slices), stained with DAPI, mounted in 70% glycerol, and then imaged with a confocal microscope (Leica, TCS SP8, Germany) or virtual microscopy slide scanning system (Olympus, VS 120, Japan).

Immunohistochemistry

Sections were washed with PBS (5 mins, 3 times), then incubated in blocking solution (10% normal goat serum and 0.3% Triton x-100 in PBS) for 1 hour at 37℃, followed by primary antibody rabbit anti-CAMKII (Abcam, ab5683, 1:500) and incubated for 72 hours at 4℃. Sections were washed with PBS (10 mins, 3 times), incubated in secondary antibody goat anti-rabbit cy3 (Jackson ImmunoResearch, 94600, 1:400) solution for 1 hour at 37℃, then washed with PBS (10 mins, 3 times), stained with DAPI and mounted with 70% glycerol.

Data Analysis

Cell counting

For counting whole brain labeled neurons using SAD-RV(ΔG)-B19(G), SAD-RV(ΔG)-N2C(G) or rAAV2-retro, the images were segmented and delineated into different brain regions with Photoshop based on the Allen Brain Atlas (http://www.brain-map.org/). The labeled neurons were quantified with ImageJ, but neurons labeled around the injection sites were not counted.

For counting CAMKII and GFP co-labeled cells, 1024 pix * 1024 pix (1183 μm * 1183 μm) images within the target brain regions were randomly selected，while for counting GAD 67 or GAD 67-GFP co-labeled cells, the brain images were segmented and delineated into different brain regions with Photoshop based on the Allen Brain Atlas. The co-labeled neurons were quantified with ImageJ.

Analysis of viral diffusion range

To analyze the diffusion range of the rAAV2-retro and the SAD-RV(ΔG)-N2C(G), the VTA injected samples containing large enough (2235 μm * 2235 μm) regions around the injection sites (referred to as CTB signals) were selected. Since viral diffusion is consecutive, the diffusion range was defined as an irregular circle centered with the CTB signals and containing consecutive GFP+ soma distributions. Only the GFP+ signals within the circle were calculated afterwards. The whole circle range was equidistantly segmented into 57.5 μm stripes centered with CTB signals along the lateral-medial or dorsal-ventral axis, respectively, using MatLab R (2014a). The GFP+ signals within each square were counted and calculated separately.

Statistical analyses

For statistical analysis of the input intensity from different brain regions, only those taking up to 1% were chosen, while for statistical analysis of the input intensity for whole brain, all regions were included. For diffusion area analysis, the percentage of signal for each square was calculated. The percentage of signal for each square was fitted to the Gaussian curve and the $W_{h/2}$ (peak width at half-height) was analyzed in order to estimate the diffusion range using MatLab R (2014a).

Independent sample t-tests， one-way ANOVA followed by LSD multiple comparison test, two-sided non-parametric test (Mann-Whitney U test) and Spearman rank correlation analysis were performed to determine statistical differences using SPSS (22.0). Statistical significance were set at ***P < 0.001, **P < 0.01 and *P < 0.05. All data values are presented as mean ± SEM. Graphs were drawn using Sigma Plot (version 10.0).

Results

SAD-RV(ΔG)-N2C(G) showed a higher retrograde infection efficiency but a

similar labeled pattern compared with SAD-RV(ΔG)-B19(G)

First, we packaged SAD-RV(ΔG)-N2C(G)-EGFP (Fig 1A, bottom), and SAD-RV(ΔG)-B19(G)-EGFP (Fig 1A, top). To compare their retrograde infection efficiencies *in vivo*, the two pseudotyped viruses were respectively mixed with CTB594 (red fluorescent signal to mark the injection sites), and injected into the Ventral Tegmental Area (VTA) of different mice (Fig 1B, Table 1). The brain samples were checked carefully to guarantee that the injection sites were restricted to the VTA (Additional file 1: Figure S1). Otherwise, the samples were excluded. For most of the GFP labeled brain regions, such as the midbrain raphe nuclei (RAmb) and the habenular neucleus (Habenular) (Fig 1C), SAD-RV(ΔG)-N2C(G)-EGFP obviously infected more neurons than SAD-RV(ΔG)-B19(G)-EGFP, even when the titer of the latter was ten times higher. The quantitative analysis of the whole brain GFP-positive neuron number is also consistent with this result (Fig 1D, $p = 5.72E-07$ for the viruses with the same titer, $p = 0.0059$ for the viruses with tenfold different titers).

The retrograde infection efficiency of SAD strain RABV was increased by pseudotyping with N2C(G), however, whether the retrograde tropism for neurons in different brain regions is also altered, remains unclear. To answer this, GFP positive neurons within each upstream brain region were quantified and normalized by dividing the total number of retrograde labeled neurons for each animal. We found that none of the analyzed brain regions showed significant differences in input percentage between SAD-RV(ΔG)-N2C(G)-EGFP and SAD-RV(ΔG)-B19(G)-EGFP groups (Additional file 2: Figure S2A). The retrograde labeled percentages in different brain regions of the two groups were highly correlated (Additional file 2: Figure S2B).

These results demonstrate that the glycoprotein gene-deleted RABV enveloped with N2C(G) could improve its retrograde infection efficiency without affecting the

retrograde tropism bias.

SAD-RV(ΔG)-N2C(G) showed a higher retrograde infection efficiency and a more restricted diffusion range compared with rAAV2-retro

The rAAV2-retro is an outstanding retrograde viral vector mainly due to its high efficiency, and has been broadly used in various functional studies of neuronal circuits [33, 42]. In order to compare the retrograde efficiency of rAAV2-retro and SAD-RV(ΔG)-N2C(G), rAAV2-retro-EF1α-EYFP was mixed with CTB594 and injected into the VTA (Fig 2A, Table 1). We found that compared to the rAAV2-retro-EF1α-EYFP with a titer of 1E+13 vg/ml, SAD-RV(ΔG)-N2C(G)-EYFP with a titer of 5E+07 IFU/ml retrograde infected more neurons (Fig 2B, 12891 ± 1080 for rAAV2-retro-EF1α-EYFP (1E+13 vg/ml); 18173 ± 2232 for SAD-RV(ΔG)-N2C(G)-EGFP (5E+07 IFU/ml), $p = 0.014$).

Given that the two viruses were tittered using different methods due to their own properties, and that the retrograde labeled neuronal number could be positively correlated with the viral titer, it is difficult to directly compare their retrograde efficiencies using different titer units. However, the increased titer could have the consequence of increasing the diffusion. Therefore, we further calculated the diffusion patterns of GFP-positive neuronal somas near the VTA. Viral labeled neuronal rates were binned every 57.5 μm along the lateral-medial or dorsal-ventral axis and fitted to the Gaussian curve. The $W_{h/2}$ was calculated to estimate the diffusion range of the two viruses. We found that compared with the rAAV2-retro-EF1α-EYFP (1E+13 vg/ml), the $W_{h/2}$ of SAD-RV(ΔG)-N2C(G)-EGFP (5E+07 IFU/ml) was much smaller along the lateral-medial axis (Fig 2C, E), but showed no significant difference along the dorsal-ventral axis (Fig 2D, F).

Together, these results suggest that SAD-RV(ΔG)-N2C(G)-EGFP had a much

higher retrograde infection efficiency and a more limited diffusion range compared to the rAAV2-retro-EF1α-EYFP.

SAD-RV(ΔG)-N2C(G) and rAAV2-retro exhibited different retrograde infection tropism in different brain regions

The rAAV2-retro was reported to be refractory to infection of certain projection neurons [33, 35]. We then compared the retrograde infection tropisms of rAAV2-retro and SAD-RV(ΔG)-N2C(G).

To achieve this, we first analyzed the GFP-positive neuron distribution patterns and proportions in different brain regions of rAAV2-retro-EF1α-EYFP and SAD-RV(ΔG)-N2C(G)-EGFP injected samples. We found that compared with the SAD-RV(ΔG)-N2C(G)-EGFP, rAAV2-retro-EF1α-EYFP preferentially labeled neurons in the MO, SS, GU, AUD, ACA, PL, ILA, ORB and AI (Fig 3A and D). Whereas, for many of the other brain regions, such as the CP, ACB, SI, BST, LHA, LPO, SNr, SCm and CS, the rAAV2-retro-EF1α-EYFP showed a quite modest infection efficiency (Fig 3B-D). It should be noted that, in the CP and ACB, which are largely enriched with GABAergic neurons and directly connected to the VTA [43-45], there were very few GFP-positive cell bodies labeled by rAAV2-retro-EF1α-EYFP. In the PAG, MRN, RAmb and a few other brain regions, there was no significant difference between the two kinds of viruses in the rate of labeled neurons (Fig 3D).

We further pooled the GFP-positive neuron signals of every individual brain region into several intact brain areas (Fig 3E) according to the Allen Brain Atlas (http://www.brain-map.org/). The data shows that in the isocortex area, the retrograde neuronal labeling rate of the rAAV2-retro-EF1α-EYFP was about 7 times higher than that of the SAD-RV(ΔG)-N2C(G)-EGFP (50.72 ± 4.85% for rAAV2-retro-EF1α-EYFP; 7.42 ± 1.47% for SAD-RV(ΔG)-N2C(G)-EGFP, $p = 0.029$). On the contrary, the SAD-

RV(ΔG)-N2C(G)-EGFP had a significantly higher percentage of labeled neurons in many of the non-cortical areas, including the striatum (0.08 ± 0.05%; 7.76 ± 1.37%; p = 0.029), amygdala (0.62 ± 0.05%; 1.44 ± 0.38%; p = 0.029), pallidum (1.30 ± 0.27%; 7.57 ± 0.86%; p = 0.029) and hypothalamus (7.27 ± 1.87%; 21.14 ± 1.56%; p = 0.029). There was no significant difference of the labeled neuron rates between the two viruses in the olfactory area (1.90 ± 0.33%; 0.69 ± 0.38%, p = 0.057), HPF/septum (0.64 ± 0.19%; 0.37 ± 0.16%; p = 0.34), claustrum/endopiriform (0.04 ± 0.05%; 0.20 ± 0.07%; p = 0.11), thalamus/epithalamus (1.81 ± 0.48%; 4.44 ± 1.25%; p = 0.11), midbrain (27.00 ± 4.07%; 35.50 ± 3.26%; p = 0.20), hindbrain(8.34 ± 0.59%; 12.43 ± 1.79%; p = 0.057) or cerebellum (0.28 ± 0.08%; 1.03 ± 0.34%; p = 0.057).

To overcome the individual variation of animals and directly compare the retrograde infection tropisms, the rAAV2-retro-EF1α-EYFP and SAD-RV(ΔG)-N2C(G)-mCherry were separately injected into the bilateral VTA in the same animal (Additional file 3: Figure S3A and B; Table 1). The differences in the labeled patterns of the two viruses were still observed in these mice (Additional file 3: Figure S3C), which is consistent with the above results (Fig 3A-D).

Since the retrograde infection efficiency or tropism biases of different brain regions may be different from the injection site, the two viruses were additionally injected into the dentate gyrus (DG), another brain region which receives a considerable amount of input from cortical areas (Fig 4A, Table 1). The representative images near the injection site show that the GFP signals labeled with the rAAV2-retro-EF1α-EYFP was much denser and diffused more widely than the SAD-RV(ΔG)-N2C(G)-EGFP (Fig 4A). Quantitative analysis of the whole brain GFP-positive neurons excluding the DG region revealed that, by injecting in the DG, the SAD-RV(ΔG)-N2C(G)-EGFP with the titer of 5E+07 IFU/ml had a comparable retrograde infection efficiency to the rAAV2-

retro-EF1α-EYFP with the titer of 1E+13 vg/ml (Fig 4E, 707 ± 70 for rAAV2-retro-EF1α-EYFP; 625 ± 241 for SAD-RV(ΔG)-N2C(G)-EGFP, p = 0.40). We further analyzed the retrograde labeled patterns of the two viruses. Since the hippocampus is a macroscopically defined cortical structure [46, 47], the hippocampus and the cortical areas were collectively referred to as an intact brain area, the HIP & Cortex. We found that, the rAAV2-retro-EF1α-EYFP significantly labeled a higher proportion of projection neurons in the HIP & Cortex (Fig 4B and F, 92.91 ± 0.74% for rAAV2-retro-EF1α-EYFP; 59.15 ± 5.28% for SAD-RV(ΔG)-N2C(G)-EGFP; P = 0.0015) compared with the SAD-RV(ΔG)-N2C(G)-EGFP. However, in the MBO (3.39 ± 0.89%; 9.75 ± 1.81%; p = 0.023) (Fig 4C and F) and MSC (0.39 ± 0.29%; 23.13 ± 3.54%; p = 0.0015) (Fig 4D and F), the rAAV2-retro-EF1α-EYFP labeled neuron rate was about 3 and 60 times lower, respectively, despite the substantial reports that the DG is densely connected to the MSC [48, 49].

The DG is also reported to receive abundant contralateral hippocampus inputs [50, 51]. To investigate the retrograde infection tropism in more detail, we also compared the labeled patterns in the contralateral hippocampus of the two viruses. The GFP-positive neuronal proportion of each contralateral subregion was calculated by normalizing the whole contralateral hippocampus inputs. We found that the retrograde labeled patterns of the two viruses in the contralateral hippocampus subregions were significantly different. The rAAV2-retro-EF1α-EYFP labeled neurons were highly enriched in the contralateral DG (99.35 ± 0.41% for rAAV2-retro-EF1α-EYFP, 27.02 ± 6.81% for SAD-RV(ΔG)-N2C(G)-EGFP; P = 0.00014), especially in the dorsal DG (Additional file 4: Figure S4A and C), but only scarcely distributed in the CA3, CA2 and CA1. However, neurons labeled with the SAD-RV(ΔG)-N2C(G)-EGFP were mainly found in the contralateral CA3 (0.33 ± 0.20% for rAAV2-retro-EF1α-EYFP,

60.51 ± 5.83% for SAD-RV(ΔG)-N2C(G)-EGFP; P = 0.00016), while also scattered in all of the DG, CA2 and CA1 regions (Additional file 4: Fig S4A and C). Since the rAAV2-retro-EF1α-EYFP labeled contralateral DG neurons were predominantly observed in the posterior ventral rather than the anterior dorsal part, we then analyzed the rostral-caudal axis distribution of the contralateral hippocampus GFP-positive neurons. We found that the rAAV2-retro-EF1α-EYFP mainly infected the caudal but not the rostral hippocampus. On the contrary, the SAD-RV(ΔG)-N2C(G)-EGFP mainly targeted neurons in the rostral hippocampus which is close to the injection site, while also slightly labeled every contralateral hippocampus part along the rostral-caudal axis (Additional file 4: Figure S4B and D).

We also established that after the virus was injected into the VTA or the DG, neurons in several regions (ACB for the VTA, MSC and contralateral CA1, CA2 and CA3 for the DG) were resistant to retrograde infection with the rAAV2-retro-EF1α-EYFP, while the SAD-RV(ΔG)-N2C(G)-EGFP could infect all of these brain regions. These results suggest that the retrograde infection biases between the rAAV2-retro-EF1α-EYFP and SAD-RV(ΔG)-N2C(G)-EGFP are quite different. The rAAV2-retro-EF1α-EYFP prefers to infect the cortical more than the subcortical neurons, while the SAD-RV(ΔG)-N2C(G)-EGFP showed a more unbiased retrograde infection tropism to neurons in different brain regions.

SAD-RV(ΔG)-N2C(G) and rAAV2-retro exhibited different efficiencies to retrograde label long-projection inhibitory neurons

The rAAV2-retro-EF1α-EYFP exhibited highly biased labeling patterns among different brain regions, that is, there was a strong preference to retrograde labeling in the long-projection cortical and hippocampal neurons, which mainly consist of excitatory subtypes [52-54], and a strong tendency to avoid labeling the brain regions

where GABAergic neurons are dominant. Therefore, we speculated as to whether the different retrograde infection patterns of the rAAV2-retro-EF1α-EYFP and SAD-RV(ΔG)-N2C(G)-EGFP were due to their tropisms toward different neuronal subtypes. To test this hypothesis, the VTA injected cortical samples were selected and immnohistochemically stained for CAMKII, a marker for excitatory neuron in the cortex [55, 56] (Fig 5A). The results evidence that, in cortical regions, the retrograde labeled neurons by both the rAAV2-retro-EF1α-EYFP and SAD-RV(ΔG)-N2C(G)-EGFP were highly co-labeled with CAMKII and showed no significant difference, although the rAAV2-retro-EF1α-EYFP group exhibited a slightly higher co-labeled tendency (Fig 5B). Thus, we further investigated the retrograde infection properties of the two viruses for the long-projection inhibitory neurons.

To achieve this, the rAAV2-retro-EF1α-mCherry and SAD-RV(ΔG)-N2C(G)-mCherry were respectively injected into the VTA of GAD67-GFP mice (Table 1). We found that, in the cortexes, none of the two viruses labeled inhibitory neurons (Fig 5C). In the ACB where rAAV2-retro showed refractory infection, about half of the SAD-RV(ΔG)-N2C(G)-mCherry labeled neurons were inhibitory (Fig 5D and H). Besides, in the other brain regions, including the ZI, LHA and PAG, the SAD-RV(ΔG)-N2C(G)-mCherry labeled a significantly higher proportion of GAD67-GFP neurons compared with the rAAV2-retro-EF1α-EYFP (Fig 5E-G, and I-K).

These results indicate that the rAAV2-retro has lower efficiency retrograde to infect long-projection inhibitory neurons compared with the SAD-RV(ΔG)-N2C(G), which may contribute to the different retrograde infection patterns of the two viruses.

Discussion

The RABV has been widely used to target long-projecting neuronal networks by either

the trans-monosynaptic spread or direct retrograde infection at the terminals. When used as a retrograde infection tracer, the RABV was largely limited by its low efficiency. Here, we found that the N2C glycoprotein derived from the RABV CVS strain was able to increase the *in vivo* retrograde infection efficiency of the SAD strain by more than tenfold. Furthermore, the pseudotyped virus SAD-RV(ΔG)-N2C(G) showed a comparable retrograde efficiency, but a more localized diffusion range and a broader tropism to different types and regions of long-projecting neuronal populations than the rAAV2-retro.

SAD-RV(ΔG)-N2C(G) are highly efficient for retrograde tracing

It has been reported that the CVS-RV(ΔG)-N2C(G) possesses higher neuronal invasiveness [57] and enhanced retrograde trans-synaptic spread [39] than the vaccine strain SAD-RV(ΔG)-B19(G). When pseudotyped with the N2C(G), SAD-RV(ΔG)-N2C(G) also exhibits increased neuro-tropism in cell culture compared with the SAD-RV(ΔG)-B19(G) [38]. However, a recent study [58] found that complementing SAD-RV(ΔG) with N2C(G) showed less or similar retrograde trans-synaptic efficiency than with B19(G). Besides, the mechanisms between retrograde trans-synaptic spread and retrograde infection at axon terminals could be different. Thus, comparison of the retrograde infection efficiency between the SAD-RV(ΔG)-B19(G) and SAD-RV(ΔG)-N2C(G) still remains obscure. Here, we addressed this conundrum in the present work. We found that after injecting the virus into the VTA, the *in vivo* retrograde infection efficiency of the SAD-RV(ΔG)-N2C(G) was much higher than that of the SAD-RV(ΔG)-B19(G) (Fig 1D). Our results are consistent with findings reported in earlier studies. Except for the enhanced neuronal invasiveness, the retrograde labeled patterns between the SAD-RV(ΔG)-N2C(G) and SAD-RV(ΔG)-B19(G) were not significantly different (Additional file 2: Figure S2). A possible explanation for the similar labeled

patterns of the two viruses is that the N2C(G) might infect the long-projecting neurons *via* the same receptors but with much higher affinity, compared with B19(G).

The rAAV2-retro is also an outstanding viral tool to efficiently target the long-projecting neurons. In this study, we found that the retrograde infection efficiency of the SAD-RV(ΔG)-N2C(G) was no less than that of rAAV2-retro (Fig 1D and 4E), but had a more restricted diffusion range (Fig 2C,E and 4A). This may be due to the different virion sizes of the viruses [6, 24, 59, 60]. Although the retrograde labeled neuronal number could be further increased with the titer of rAAV2-retro, this could lead to a larger viral diffusion range, which subsequently raises the risk of non-specific infection of neurons upstream to the brain regions adjacent to the injected site. With the comparable retrograde efficiency to the rAAV2-retro, the more localized diffusion range of the SAD-RV(ΔG)-N2C(G) could make it more suitable to target the input neuronal circuit of small nuclei.

The recently developed receptor complementation strategy for CAV-2 has also extensively improved its retrograde transport efficiency and overcome the limitation of biased tropism [32]. However, this strategy needs an additional viral injection prior to the CAV-2-Cre, which complicates animal surgery, and could be hard to access the difficult-to-inject areas or target large volume or even the whole-brain upstream tissues. The SAD-RV(ΔG)-N2C(G), for its intrinsic high efficiency and broad tropism, should be able to overcome these limitations. However, further studies analyzing the efficiencies of the CAV-2 and the SAD-RV(ΔG)-N2C(G) in different neuronal circuits should also be very valuable in guiding the applications of the these viral tools.

The retrograde neuronal tropism biases of rAAV2-retro and SAD-RV(ΔG)-N2C(G) are rather different

The present study showed that the rAAV2-retro and SAD-RV(ΔG)-N2C(G), when

injected either in VTA or DG, revealed different retrograde labeling patterns. Some brain regions, especially the striatum and basal forebrain, were largely resistant to the retrograde infection of the rAAV2-retro, but susceptible to SAD-RV(ΔG)-N2C(G) (Fig 3B and 4D), showing that the SAD-RV(ΔG)-N2C(G) has a broader retrograde tropism to long-projecting neurons.

Our experimental data on the selectivity of SAD-RV(ΔG)-N2C(G) and rAAV2-retro toward the two most dominant neuron types, CAMKII+ and GAD67+, showed that few neurons labeled with rAAV2-retro are long-projecting GABAergic, while a significant percentage of the SAD-RV(ΔG)-N2C(G) labeled neurons (from 17 to 50 in the subcortical regions) is long-projecting GABAergic (Fig 5D-K). However, it should be noted that the majority of the long-projecting neurons in the cortex are excitatory [52, 53, 61] (Fig 5A and B). Therefore, the distinct retrograde infection preference of the two viruses over excitatory and inhibitory long projection neurons generate different input patterns for a given brain region. The VTA receives extensive subcortical neuronal innervations [62, 63], while the major inputs of the DG are from the cortical and hippocampal subregions [50, 51]. Indeed, the input patterns of either the VTA or the DG, revealed by the two viruses, are rather different (Fig 3D, E, 4F, Additional file 4: Figure S4C and D). The significantly different neuronal tropisms of these results suggest that when we interpret our tracing data, the viral tools should be considered carefully. In fact, RABV may also exhibit tropism biases in some cases [64]. Thus, the best selection of viral tools should depend on the specific circuit being studied. And the combined use of multiple viral tracers should get us closer to the ground truth.

Our finding that the rAAV2-retro showed much higher efficiency to label the long-projecting cortical and hippocampal excitatory neurons is partially consistent with previous reports, which compared the properties of the SAD-RV(ΔG)-B19(G) and

rAAV2-retro [37].

Although we demonstrated that the SAD-RV(ΔG)-N2C(G) has high efficiency and broad tropism for retrograde labeling of neuronal circuits, further work is still required to overcome the viral cytotoxicity for achieving long term functional studies or transgenic expression. Fortunately, recently developed strategies which delete certain genes [35] or introduce self-inactivating vector [37] of the SAD-RV(ΔG) have successfully overcome the limitation. One might argue that CVS-RV(ΔG)-N2C(G) has decreased toxicity more than SAD-RV(ΔG)-N2C(G) and hence is superior. However, since the viral production is much easier for SAD-RV(ΔG) than for CVS-RV(ΔG), and these most powerful designs [35, 37] to reduce the toxicity of RABV to date are based on the SAD-RV(ΔG) vector, our introduction of the SAD-RV(ΔG)-N2C(G) and our ongoing research to integrate these systems could provide more easily accessible and promising retrograde tools for the community.

Conclusion

We have provided experimental evidence for a powerful viral tool, the N2C(G) enveloped SAD-RV(ΔG)-N2C(G), which has high retrograde infection efficiency and broad neuro-tropism to target the input neuronal circuits. The comparison of the infection efficiency and neuro-tropism of SAD-RV(ΔG)-N2C(G) and rAAV2-retro provide valuable information for the selection of these two viruses in individual research.

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Conflict of Interest

There is no interest of conflict associated with the study of any of the authors.

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Table

Table 1. Experimental parameters for retrograde tracing.

Animal	Number	Nucleus	Virus	Dose (IFU/mL or vg/mL)	Vol. (μl)	Injection (Day)	Perfusion (Day)
C57BL/6	4	VTA	SAD-RV(ΔG)-B19(G)-EGFP	5.00E+07	0.2	Day1	Day7
C57BL/6	4	VTA	SAD-RV(ΔG)-B19(G)-EGFP	5.00E+08	0.2	Day1	Day7
C57BL/6	4	VTA	SAD-RV(ΔG)-N2C(G)-EGFP	5.00E+07	0.2	Day1	Day7
C57BL/6	4	VTA	rAAV2-retro-Ef1α-EYFP	1.00E+13	0.2	Day1	Day21
C57BL/6	4	VTA(Right)	rAAV2-retro-Ef1α-EYFP	1.00E+13	0.2	Day1	Day21
		VTA(Left)	SAD-RV(ΔG)-N2C(G)-mCherry	1.10E+08	0.2	Day14	
C57BL/6	4	DG	SAD-RV(ΔG)-N2C(G)-EGFP	5.00E+07	0.1	Day1	Day7
C57BL/6	3	DG	rAAV2-retro-Ef1α-EYFP	1.00E+13	0.1	Day1	Day21
GAD67-GFP	4	VTA	SAD-RV(ΔG)-N2C(G)-mCherry	1.10E+08	0.2	Day1	Day7
GAD67-GFP	3	VTA	rAAV2-retro-Ef1α-mCherry	1.68E+13	0.2	Day1	Day21

Table 2. Abbreviations of brain areas

Brain areas	Abbreviations	Brain areas	Abbreviations
Somatomotor areas	MO	Bed nuclei of the stria terminalis	BST
Anterior cingulate area	ACA	Dorsomedial nucleus of the hypothalamus	DMH
Prelimbic area	PL	Posterior hypothalamic nucleus	PH
Somatosensory areas	SS	Ventromedial hypothalamic nucleus	VMH
Gustatory areas	GU	Lateral hypothalamic area	LHA
Auditory areas	AUD	Lateral preoptic area	LPO
Infralimbic area	ILA	Zona incerta	ZI
Orbital area	ORB	Ventral tegmental area	VTA
Agranular insular area	AI	Superior colliculus, motor related	SCm
Retrosplenial area	RSP	Periaqueductal gray	PAG
Hippocampal region	HIP	Substantia nigra, reticular part	SNr
Retrohippocampal region	RHP	Midbrain reticular nucleus	MRN
Field CA1	CA1	Pretectal region	PRT
Field CA2	CA2	Midbrain reticular nucleus, retrorubral area	RR
Field CA3	CA3	Midbrain raphe nuclei	RAmb
Dentate gyrus	DG	Substantia nigra, compact part	SNc
Caudoputamen	CP	Pedunculopontine nucleus	PPN
Nucleus accumbens	ACB	Parabrachial nucleus	PB
Olfactory tubercle	OT	Pontine reticular nucleus, caudal part	PRNc
Substantia innominata	SI	Pontine reticular nucleus	PRNr
Medial septal complex	MSC	Superior central nucleus raphe	CS
Habenular nucleus	Habenular	Cerebellar nuclei	CBN
Mammillary body	MBO		

Figure legends

Fig 1. The retrograde infection efficiency of SAD-RV(ΔG)-N2C(G)-EGFP is higher than that of SAD-RV(ΔG)-B19(G)-EGFP. A: Schematic for the virion structures of SAD-RV(ΔG)-B19(G)-EGFP (top) and SAD-RV(ΔG)-N2C(G)-EGFP (bottom). B: Schematic for the *in vivo* tracing study. Low dosage of CTB594 was respectively co-

injected with low titer SAD-RV(ΔG)-B19(G)-EGFP (5E+07 IFU/mL, top), high titer SAD-RV(ΔG)-B19(G)-EGFP (5E+08 IFU/mL, middle) or SAD-RV(ΔG)-N2C(G)-EGFP (at a titer of 5E+07 IFU/mL, bottom) into the VTA in C57 mice. C: A large number of upstream brain regions of the VTA, such as the habenular nucleus (Habenular, left) and the midbrain raphe nuclei (RAmb, right), were retrograde labeled by SAD-RV(ΔG)-N2C(G)-EGFP (bottom) or SAD-RV(ΔG)-B19(G)-EGFP with different titers (top and middle). D: The whole brain retrograde infected neuronal numbers by low titer SAD-RV(ΔG)-B19(G)-EGFP (5E+07 IFU/mL), high titer SAD-RV(ΔG)-B19(G)-EGFP (5E+08 IFU/mL) and SAD-RV(ΔG)-N2C(G)-EGFP (5E+07 IFU/mL). Scale bar: 200 μm. n = 4 mice for each group, one-way ANOVA followed by LSD multiple comparison test, *p < 0.05, **p < 0.01, ***p < 0.001. n.s., no significant difference. The nuclei were stained in blue by DAPI.

Fig 2. The comparison of retrograde infection efficiency and diffusion range between SAD-RV(ΔG)-N2C(G) and rAAV2-retro. A: Representative image illustrates the injection site of rAAV2-retro-EF1α-EYFP in the VTA. CTB594 was co-injected with the virus to delineate the injection site. B: The whole brain retrograde infected neuronal numbers by SAD-RV(ΔG)-N2C(G)-EGFP (5E+07 IFU/mL) and rAAV2-retro-EF1α-EYFP (1E+13 IFU/mL). C-D: The lateral-medial (C) or dorsal-ventral (D) diffusion pattern of the rAAV2-retro-EF1α-EYFP (green dots and line) and SAD-RV(ΔG)-N2C(G)-EGFP (red dots and line) around injection sites. The signal percentages distributed along the lateral-medial or dorsal-ventral axis of all animals were fitted to the Gaussian curve. $W_h/_2$ represents peak width at half-height. E-F: Statistic analysis of the lateral-medial (E) or dorsal-ventral (F) diffusions of rAAV2-retro-EF1α-EYFP and SAD-RV(ΔG)-N2C(G)-EGFP. The $W_h/_2$ of each animal (n = 4 mice for each virus) was calculated and compared between the two viral groups. T TEST. *p < 0.05, **p < 0.01, ***p < 0.001. n.s., no significant difference. Scale bar: 200 μm.

Fig 3. The retrograde infection tropism biases of SAD-RV(ΔG)-N2C(G) and rAAV2-retro injected in the VTA. A-C: Representative images show the different retrograde labeled neuron patterns with rAAV2-retro-EF1α-EYFP (top) or SAD-RV(ΔG)-N2C(G)-EGFP (bottom) in different brain regions, including the cortex (A), the striatum (B) and the LHA (C). D: Quantitative analysis of the input proportions of different nuclei labeled with SAD-RV(ΔG)-N2C(G)-EGFP and rAAV2-retro-EF1α-EYFP. For most of the nuclei, the input proportions of the neurons labeled with the two viruses are dramatically different. E: The input proportions of intact brain areas pooled from the discrete nuclei in D. Compared with SAD-RV(ΔG)-N2C(G)-EGFP, rAAV2-retro-EF1α-EYFP preferentially infected the isocortex, but rarely infected the striatum, amygdala, palidum and hypothalamus. n = 4 mice for each group, Mann-Whitney U test. *p < 0.05, **p < 0.01, ***p < 0.001. Scale bar: 200 μm.

Fig 4. The different retrograde labeled patterns of SAD-RV(ΔG)-N2C(G)-EGFP and rAAV2-retro-EF1α-EYFP injected in the hippocampus. A: Coronal sections near the injection sites (indicated by the red signals of CTB594) in the DG by the two viruses (rAAV2-retro-EF1α-EYFP, top; SAD-RV(ΔG)-N2C(G)-EGFP, bottom). B-D: Representative images reveal that the retrograde labeled patterns with SAD-RV(ΔG)-N2C(G)-EGFP and rAAV2-

retro-EF1α-EYFP are quite different in many brain regions, such as in the hippocampus (B), the MBO (C) and in the MSC (D). E: The whole brain retrograde infected neuronal numbers with SAD-RV(ΔG)-N2C(G)-EGFP and rAAV2-retro-EF1α-EYFP. F: The input proportions of different areas. n = 3 mice for rAAV2-retro-EF1α-EYFP; n = 4 mice for SAD-RV(ΔG)-N2C(G)-EGFP. T-Test. *p < 0.05, **p < 0.01, ***p < 0.001. Scale bar: 50 μm for the magnified panels in B, 200 μm for panels A-D.

Fig 5. rAAV2-retro and SAD-RV(ΔG)-N2C(G) exhibited different efficiencies in retrograde labeling of long-projection inhibitory neurons. A: Representative cortical images display the colocalization (merged, left) of viral labeled neurons (green, middle) and immunofluorescent CAMKII staining (red, right). B: The CAMKII positive rates of GFP labeled neurons with SAD-RV(ΔG)-N2C(G)-EGFP and rAAV2-retro-EF1α-EYFP in the cortical regions. Mann-Whitney U test, n.s., no significant difference. C-G: Representative images indicate the colocalization of GAD67-GFP (green, GAD 67-GFP mice) and viral labeled neurons with the rAAV2-retro-EF1α-mCherry (red, top) or SAD-RV(ΔG)-N2C(G)-mCherry (red, bottom) in the Cortex (C), ACB (D), ZI (E), LHA (F) and PAG (G). H-K: Quantitative analysis of co-labeled rates of GAD67-GFP with viral labeled red neurons. The SAD-RV(ΔG)-N2C(G)-mCherry labeled more GAD67-GFP positive neuronal rates than the rAAV2-retro-EF1α-mCherry in the ACB (H), ZI (I), LHA (J) and PAG (K). n = 3 mice for rAAV2-retro-EF1α-mCherry. n = 4 mice for SAD-RV(ΔG)-N2C(G)-mCherry. T-Test. *p < 0.05, **p < 0.01, ***p < 0.001. Scale bar: 200 μm.